ehp ENVIRONMENTAL HEALTH PERSPECTIVES
http://www.ehponline.org

TNFα and IL-6 Responses to Particulate Matter *in Vitro*: Variation According to PM Size, Season, and Polycyclic Aromatic Hydrocarbon and Soil Content

Natalia Manzano-León, Jesús Serrano-Lomelin, Brisa N. Sánchez, Raúl Quintana-Belmares, Elizabeth Vega, Inés Vázquez-López, Leonora Rojas-Bracho, Maria Tania López-Villegas, Felipe Vadillo-Ortega, Andrea De Vizcaya-Ruiz, Irma Rosas Perez, Marie S. O'Neill, and Alvaro R. Osornio-Vargas

http://dx.doi.org/10.1289/ehp.1409287

Received: 1 October 2014
Accepted: 10 September 2015
Advance Publication: 15 September 2015

Environ Health Perspect DOI: 10.1289/ehp.1409287
Advance Publication: Not Copyedited

TNFα and IL-6 Responses to Particulate Matter *in Vitro*: Variation According to PM Size, Season, and Polycyclic Aromatic Hydrocarbon and Soil Content

Natalia Manzano-León[1], Jesús Serrano-Lomelin[2], Brisa N. Sánchez[3], Raúl Quintana-Belmares[1], Elizabeth Vega[4,5], Inés Vázquez-López[1], Leonora Rojas-Bracho[6], Maria Tania López-Villegas[6], Felipe Vadillo-Ortega[7], Andrea De Vizcaya-Ruiz[8], Irma Rosas Perez[9], Marie S. O'Neill[3], and Alvaro R. Osornio-Vargas[1,10]

[1]Instituto Nacional de Cancerología. México, D.F., México; [2]School of Public Health, University of Alberta, Edmonton, AB, Canada; [3]Departments of Environmental Health Sciences and Epidemiology, University of Michigan, School of Public Health. Ann Arbor, Michigan, USA; [4]Instituto Mexicano del Petróleo, México, D.F., México; [5]Instituto Nacional de Investigaciones Nucleares, Estado de México, México; [6]Instituto Nacional de Ecología y Cambio Climático, México, D.F., México; [7]UNAM at the National Institute of Genomic Medicine, México, D.F., México; [8]Departamento de Toxicología, Cinvestav, México, D.F., México; [9]Centro de Ciencias de la Atmósfera, UNAM, México, D.F., México; [10]Department of Paediatrics, University of Alberta, Edmonton, AB, Canada

Address correspondence to Alvaro R. Osornio-Vargas, Department of Paediatrics, University of Alberta, 3-591 ECHA, 11405 87th Ave, Edmonton, T6G1C9, Canada. Telephone (780) 492-7092. E-mail: osornio@ualberta.ca

Running title: Seasonal PAHs and PM-induced TNFα secretion

Acknowledgments: We thank the Gobierno del Distrito Federal for facilitating air sampling. We thank José Garcia-Yee, Sergio Escalona, Martha Hernández-Guzmán, Nalleli Reyes-Martínez, Laura Sevilla-Tapia, Jesús Valencia, Fernando Vega-Bernal, Leticia Martínez and Eva Salinas for technical assistance. Chemical analysis was performed in the Instituto Mexicano del Petróleo (IMP-4278). This project was supported by: Fogarty International Research Collaboration Award; the National Institute for Environmental Health Sciences (R01-ES016932 and R01-ES017022-01); and the Instituto Nacional de Ecología.

Competing financial interests: The authors have no conflict of interest to declare.

ABSTRACT

Background: Observed seasonal differences in particulate matter (PM) associations with human health may be due to composition and toxicity seasonal interactions.

Objectives: Assess seasonality in PM composition and *in vitro* PM pro-inflammatory potential using multiple PM samples.

Methods: We collected ninety weekly PM_{10} and $PM_{2.5}$ samples during the rainy-warm and dry-cold seasons in five urban areas with different pollution sources. The elements, polycyclic aromatic hydrocarbons (PAHs) and endotoxins characterized in samples were subjected to principal component analysis (PCA). We tested the PM potential to induce TNFα and IL-6 secretion in cultured human monocytes (THP-1) and modeled pro-inflammatory responses using component scores.

Results: PM composition varied by size and season. PCA identified two main components that varied by season. Component 1 (C_1) grouped combustion-related constituents (e.g. V, benzo(a)pyrene, benzo(a)anthracene). Component 2 (C_2) grouped soil-related constituents (e.g. endotoxins, Si, Al). PM from the rainy-warm season was high in C_2. PM **(**especially $PM_{2.5}$) from the dry-cold season was rich in C_1. Higher levels of cytokine production related with PM_{10} and C_2 (rainy-warm season), whereas $PM_{2.5}$ and C_1 were associated with lower levels (dry-cold season). TNFα secretion was higher following exposure to PM with high (versus low) C_2 content, but TNFα secretion in response to PM was decreased following exposure to samples containing ≥0.1% of C_1-related PAHs, regardless of C_2 content. IL-6 results suggest more complex interactions between PM components and particle size.

Conclusions: Variations in PM soil and PAHs content underlie seasonal and PM size related patterns in TNFα secretion. These results suggest that the mixture of components in PM explains some seasonal differences in associations between health outcomes and PM in epidemiologic studies.

INTRODUCTION

Exposure to particulate matter (PM) has been associated with cardiorespiratory diseases and adverse birth outcomes, among other endpoints (Pope and Dockery 2006; Shah and Balkhair 2011; Zanobetti et al. 2014). PM is a complex mixture of several components including carbon, metals, organic compounds, ions and biological elements (Pope and Dockery 2006). Seasonal variability in PM chemical composition is reported for 1) the content of organic carbon, nitrates and sulfates in samples collected during five years in 187 counties of the USA (Bell et al. 2007); 2) endotoxin in studies performed in Turin (Traversi et al. 2010); 3) nickel (Ni), copper (Cu), zinc (Zn) and lead (Pb) in Venice (Toscano et al. 2011); and 4) coarse PM enrichment in water-soluble organic carbon and nitrate during the summer in Los Angeles (Cheung et al. 2011).

Evidence also links seasonal changes in PM levels and chemical composition with health outcomes. Associations between carbon monoxide (CO), sulfur dioxide (SO_2) and PM levels and increased mortality differed according to season in two metropolitan areas in the USA (Moolgavkar 2003). Mortality was higher in association with specific characteristics of PM in Arizona during the spring and summer months (Smith et al. 2000) and similar seasonal increments in summer PM-related mortality were observed in cities from the northeastern USA (Peng et al. 2005).

Simultaneous evaluation of chemical composition of PM collected in different seasons may help elucidate the relationship between specific constituents of PM and biological effects. Becker et al. (2005) reported that North Carolina PM samples collected in the autumn were richer in iron (Fe), silicon (Si) and chromium (Cr) and had a higher pro-inflammatory potential than PM samples collected in other seasons. Another study showed a relation between higher

levels of PM_{10} (particulate matter with mean aerodynamic diameter ≤10 μm) with a high content of Saharan desert dust and increased mortality, usually between spring and autumn (Diaz et al. 2012). Mugica et al. (2010) found that seasonal variation in the PM_{10} content of polycyclic aromatic hydrocarbons (PAHs) in Mexico City was related with differences in toxicity.

We analyzed PM_{10} and $PM_{2.5}$ (particulate matter with mean aerodynamic diameter ≤2.5 μm) season-specific composition and *in vitro* pro-inflammatory potential, using samples collected in the rainy-warm (summer) and dry-cold (winter) seasons in five areas of Mexico City with different urban activities. We used ninety samples to assess variability in composition and cell responses applying multivariate analysis and regression modeling, an approach not conventionally used in toxicological studies. This work is part of an ongoing epidemiological study of pregnant women's exposure to air pollution and birth outcomes in Mexico City (O´Neill et al. 2013).

METHODS

PM Sampling

PM_{10} and $PM_{2.5}$ were collected in five sites of Mexico City, using High-Vol samplers and nitrocellulose membranes (Manzano-Leon et al. 2013). Samples were collected weekly between May-August 2009 and November 2010-March 2011, corresponding to the rainy-warm and dry-cold seasons of the year, respectively. Sampling sites were selected according to their proximity to the official sites of the Mexico City's air monitoring network and were located in areas mainly representing industrial, business, and residential activities. The selected residential areas vary in traffic related pollution, demographics and urban infrastructure (INE-SEMARNAT 2011).

PM was mechanically recovered from the membranes (Alfaro-Moreno et al. 2009). Weekly PM samples were pooled by month, site and size, resulting in 40 samples from the rainy-warm season and 50 from the dry-cold season. We determined the chemical composition (elements, PAHs and endotoxins) and pro-inflammatory potential (TNFα and IL-6 production) for each sample.

Chemical analysis

Elements

Inductively Coupled Plasma Mass Spectrometry (Agilent 7500a) determined 33 elements. PM samples (~1 mg) were dissolved in 3 mL of deionized water (18.2MΩ/cm). Prior to analysis, samples were filtered using nylon membranes (0.2 μm; Millipore GNWP). The analysis was conducted using a flow rate of 1.0 L/min of argon, an incident power of 1.39 kW, a radiofrequency of 1.76 V, and a discriminator of 9.5 mV. Elements with atomic number from lithium (Li) to Pb were subject to three scans, 100 passes, using 32 channels. Interference equations were used for corrections in all samples (see Vega et al. 2011 for details).

Polycyclic aromatic hydrocarbons (PAHs)

Sixteen PAHs, commonly assessed in other studies (Camatini et al. 2012; Derghman et al. 2012) were identified using High Performance Liquid Chromatography (Agilent HP, 1100 series) and a Nucleosil column (Macherey-Nagel, 265 mm, 100-5C18PAH), with an automatic sample injector and a fluorescent detector. PM samples (~1 mg) were extracted with 30 mL of dichloromethane in a microwave oven (CEM, model MarsX). The extracts were concentrated to 1.0 mL under a gentle stream of ultrapure nitrogen supplied by a nitroevaporator. They were then

changed into 4.0 mL of acetonitrile and reconcentrated to 0.5 mL with ultrapure nitrogen. The validation method parameters were: linearity (R^2>0.98), accuracy/precision (RSD<3%), detection limit (0.004 μg/mL). Quantification limits and percent recovery, depending on the compound, were 0.01 to 0.03 μg/mL and 60.2 to 94.2%, respectively.

Endotoxins

Endotoxins were extracted from PM samples (1 mg/mL) using Tris buffer 50 mM (Lonza) sonicated for one hour at 22°C and vortexed every minute during 15 minutes. 500 μL aliquots from each extract were used for serial dilutions (1:5) to identify an optimal dilution. All samples were handled in glass tubes and plastic materials free of endotoxins. The endotoxins analysis was conducted using kits for the kinetic chromogenic Limulus Amebocyte Lysate (LAL) assay with a Kinetic-QCL Reader and Software (Lonza Kinetic-QCL), following the supplier's specifications. The samples were analyzed in duplicate in 96-well microplates. Endotoxins concentration was determined using *Escherichia coli* O55:B5 endotoxin as a standard (10 endotoxin units per ng) and endotoxins content was expressed as ng/mg of particles. The reported endotoxins content represents the overall activity of various endotoxin types potentially present in the samples.

Pro-inflammatory cytokines (TNFα and IL-6)

We selected TNFα and IL-6 as the cytokines of interest, due to their known participation as acute response factors in PM-mediated pro-inflammatory responses (Hiraiwa and van Eeden, 2013). To evaluate *in vitro* cytokine responses, we used THP-1 cells (human monocytic cell line), obtained from the American Type Culture Collection. Cells were cultured in 10% fetal

bovine serum-RPMI (Cat. A10491, SIGMA) containing penicillin (50 U/mL)/streptomycin (50 μg/mL). Cultures were kept at 37°C in a 5% CO_2/95% air atmosphere.

TNFα and IL-6 were measured in the supernatants of 550,000 cells/mL exposed during 24 hours to 80 μg/mL of PM_{10} or $PM_{2.5}$. All exposures were conducted in 24 well plates maintaining an equivalency of 80 μg/mL to 40 μg/cm^2. We designed the study considering a fixed PM mass concentration in order to allow the PM constituents' concentrations to guide the concentration-dependent responses. We anticipated that PM constituents' concentrations would vary according to the size, site and season. Previous work identified 80 μg/mL as the optimal concentration to induce cytokine production with minimal loss in cell viability (up to10%) (Manzano-León et al. 2013). Cells were kept in serum free media 24 hours before PM exposure. Fresh PM aliquots (1 mg/mL) were prepared, vortexed and sonicated, immediately before each exposure. Subsequently, ELISA (InVitrogen) determined TNFα and IL-6 in the supernatants. We conducted three independent experiments with each PM sample. Each experiment represented the average concentration of duplicates. Non-exposed cells were used as negative controls and their basal cytokine produced levels (TFNα = 3.225 pg/mL and mean IL-6 = 1.135 pg/mL) were subtracted from the experimental values. Cells exposed to 10 μg/mL lipopolysaccharide (LPS) served as positive controls. The results are expressed in pg/mL.

Statistical Analysis.

The analysis only included those constituents identified in all samples. Medians, means and 95% confidence intervals (CIs) were tabulated for the PM_{10} and $PM_{2.5}$ constituents and groups of constituents (endotoxins, PAHs and elements). Seasonal medians were compared using the Mann-Whitney test. Medians, means and percentages for each PM_{10} and $PM_{2.5}$ constituent

were calculated, and medians were compared between seasons by Mann-Whitney tests. All constituents were natural log-transformed (ln) and Principal component analysis (PCA) was used to group the multiple PM constituents into components according to the correlations among constituents. The scree plot criterion was used to choose the number of components to retain (Afifi et al. 2004). Component scores were computed and compared between seasons according to PM-size using ANOVA and also plotted using different symbols to visualize seasonal patterns of PM composition by PM-size.

Similarly, we obtained medians, means and 95% CIs of TNFα and IL-6 levels by season and PM-size.

Associations between the principal components and the cytokines (ln) were calculated using partial correlation coefficients (*r)* controlling for PM-size. The relative participation of the PAHs in C_1 (C_1-related PAHs) by PM-size and season was calculated as the percentage of the total content of the constituents grouped in C_1 and C_2 (C_1+C_2 content). Regression models adjusting for PM-size and C_1+C_2 content evaluated the relationship between the percentage of C_1-related PAHs and cytokine production (ln). C_1+C_2 content was categorized in quartiles: Low; Low-Medium; Medium-High, and High. All statistical analysis used SPSS_v20 and STATA_v.10.

RESULTS

Seasonal characteristics

The dry-cold season had the following five-site averages: temperature 14.8±0.98^{o}C, relative humidity 39.42±2.92%, accumulated precipitation 22±5 mm, one hour maximum ozone

0.073±0.026 ppm, PM_{10} 67.8±14.02 μg/m^3, and $PM_{2.5}$ 31.2±2.59 μg/m^3. Values for the rainy-warm season were: temperature of 18.6±0.89°C, relative humidity of 57±2%, accumulated precipitation 660±78 mm, one hour maximum ozone 0.086±0.027 ppm, PM_{10} 42.6±7.09 μg/m^3, and $PM_{2.5}$ 21±2 μg/m^3 (SEDEMA, 2015).

PM chemical composition

Overall, 32 PM constituents (22 elements, 9 PAHs and endotoxins) were found in all samples (see Supplemental Material, Tables S1 and S2) and summarized in Table 1. Constituents not represented in all samples and excluded from the analysis are in Supplemental Material, Table S3. The percentage of the total PM mass explained by the measured constituents was 6.8% (68.45 μg/mg) in the dry-cold season PM_{10}; 12.6% (126.25 μg/mg) in the rainy-warm season PM_{10}; 4.7% (46.60 μg/mg) in the dry-cold season $PM_{2.5}$ and 2.8% (27.69 μg/mg) in the rainy-warm season $PM_{2.5}$.

Individual PM-constituents varied by season and PM-size (see Supplemental Material, Tables S1 and S2). On average, the PM_{10} elemental constituents' concentration was higher in the rainy-warm season than in the dry-cold (126,206 ng/mg vs. 68,391 ng/mg). Calcium (Ca), sulfur (S), potassium (K), sodium (Na), magnesium (Mg), Si, Fe, aluminum (Al), and Zn accounted for 98% of the total elemental concentration in PM_{10} from both seasons, and seven of these elements (Ca, S, K, Na, Si, Fe, and Al) were significantly different between seasons ($p<0.05$) (see Supplemental Material, Table S1). Median concentrations of PAHs in PM_{10} were slightly higher in the dry-cold season ($p=0.052$) (Table 1). Average PM_{10} endotoxins concentrations were no different between seasons ($p=0.584$).

The average elemental constituent concentrations in $PM_{2.5}$ were higher in the dry-cold season than in the rainy-warm season (46,529 ng/mg vs. 27,657 ng/mg). The content of S, Ca, K, Na, Zn, Mg, Fe, Cu, vanadium (V), Al and Si accounted for 98% of the elemental concentration in $PM_{2.5}$ samples from the dry-cold season, and Ca, S, Na, K, Zn, Mg, Si and Cu in $PM_{2.5}$ samples from the rainy-warm season (see Supplemental Material, Table S2). Median concentrations of PAHs in $PM_{2.5}$ samples were higher in the dry-cold season than in the rainy-warm season (56.7 ng/mg vs. 24.2 ng/mg; p=0.001) (Table 1). Endotoxins were significantly higher in $PM_{2.5}$ from the dry-cold than in $PM_{2.5}$ from the rainy-warm season (2.1 ng/mg vs. 0.5 ng/mg; p=0.001).

PM elements dominated the mass explained by the measured PM constituents, making up to 99.97% of that fraction. Although the percentage of the PM mass explained by PAHs was <1%, it was nearly three times greater in the dry-cold season than in the rainy-warm season when combining both PM-sizes (0.11% vs. 0.04%). Generally, endotoxins content was higher in PM_{10} than in $PM_{2.5}$, but only $PM_{2.5}$ showed seasonal differences (p=0.001) (Table 1). During the rainy-warm season the mean concentration of all measured constituents was 4.6 times higher in PM_{10} than in $PM_{2.5}$ (126,250 ng/mg vs. 27,690 ng/mg), while in the dry-cold season, the relation was 1.5 times higher (68,450 ng/mg vs. 46,602 ng/mg) (see Supplemental Material, Tables S1 and S2).

Principal component analysis

PCA was performed including endotoxins, PAHs (acenaphthylene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, fluoranthene and pyrene), and elements (S, Ca, K, Na, Zn, Mg, Fe, Cu, V, Al, and Si) that

accounted for 98% of all elements concentration, lowering the number of explanatory variables while maximizing the case/variable ratio (n>>number of variables) (Legendre and Legendre, 2012).

Three principal components were extracted, explaining 74.3% of total variance (see Supplemental Material, Table S4). Component 1 (C_1) explained 43.4% of total variance and was mainly comprised of K, V, S, benzo(a)pyrene, benzo(a)anthracene, fluoranthene, chrysene, benzo(k)fluoranthene, benzo(g,h,i)perylene, Fe, Zn and benzo(b)fluoranthene. Component 2 (C_2) accounted for 21.5% of total variance and was mainly comprised of Ca, Mg, endotoxins, Si, Al and Na. Finally, Component 3 (C_3) mainly comprised of pyrene and acenaphthylene and explained 9.4% of total variance.

Seasonal differences of component scores according to PM-size were estimated. PM_{10} samples from the rainy-warm season had higher levels of C_2 and C_3 ($p<0.05$) and lower C_1 levels ($p<0.05$) than the samples from the dry-cold season. $PM_{2.5}$ samples showed significant seasonal differences exclusively for C_1, which was higher in the dry-cold season ($p<<0.05$) (see Supplemental Material, Figure S1).

Content distribution of C_1 and C_2 by size and season of the 90 PM samples had four distinct patterns: 1) $PM_{2.5}$ from the rainy-warm season had low C_1 and C_2 values, 2) PM_{10} from the dry-cold season grouped around average C_1 content and just above average C_2 content, 3) PM_{10} from the rainy-warm season had average C_1 content and high C_2 content, and 4) $PM_{2.5}$ from the dry-cold season grouped in the low C_2 high C_1 values (Figure 1).

Levels of TNFα and IL-6 induced by PM_{10} and $PM_{2.5}$

The cytokine responses obtained from the 90 PM_{10} and $PM_{2.5}$ samples significantly varied by season and size ($p<0.05$) (Table 2). Both PM-size samples from the rainy-warm season produced a more potent average cell response (TNFα and IL-6) than those from the dry-cold season. TNFα secretion was much higher and more homogeneous than that of IL-6 across different PM exposures (coefficients of variation for TNFα according to season and PM size ranged from 22.5% to 125.4%; for IL-6 they ranged from 59.4% to 243.8%) (Table 2). PM_{10} exhibited more potent TNFα and IL-6 responses than $PM_{2.5}$ in both seasons.

Partial correlation coefficients between principal components and cytokines levels (ln) indicated that C_1 had a statistically significant negative correlation with TNFα (r=-0.32; p=0.002) and IL-6 (r=-0.36; $p<0.001$), whereas C_2 had a statistically significant positive correlation with both cytokines (TNFα: r=0.65; $p<0.001$; IL-6: r=0.5; $p<0.001$). However, the C_1 and TNFα scatter plot showed an inverse U-shape relationship, such that TNFα secretion in response to PM exposure was highest when C_1 content was in the middle of the distribution, and lowest in response to PM with very low and very high C_1 content (Figure 2A). Those extreme values corresponded to $PM_{2.5}$ samples from rainy-warm and dry-cold seasons, respectively. In contrast, the scatter plot and linear regression of C_2 and TNFα values showed a statistically significant positive correlation (r=0.65; $p<0.001$) (Figure 2B). Associations of IL-6 with C_1 and C_2 were similar to corresponding associations with TNFα; however the patterns were less clear due to more dispersed data (Figures 2C and 2D). C_3 was not associated with either one of the cytokines (p-value = 0.407 for TNFα and 0.257 for IL-6) and further analysis was not pursued (data not shown).

TNFα was negatively associated with C_1-related PAHs (Figure 3A and 3B). Furthermore, based on a regression model adjusted for PM-size and C_1+C_2 content, the percentage of PAHs present in C_1 (C_1-related PAHs) was negatively associated with TNFα production (ln) in response to PM exposure (regression coefficient –8.21; 95% CI –10.7, –5.7 for the % C_1-related PAHs) (see Supplemental Material, Table S5). In addition, PM-size (PM_{10}>$PM_{2.5}$) and C_1+C_2 content (concentration dependent) were significant positive predictors of TNFα production in response to PM exposure. When the proportion of C_1-related PAHs was larger than 0.1%, PM-induced TNFα levels were low regardless of the C_1+C_2 content, as better seen with untransformed TNFα values (Figure 3B). For IL-6, the model was not statistically significant (data not shown), probably as a result of data dispersion and the number of observations with very low IL-6 concentration values (Figure 2C).

DISCUSSION

We described the inflammatory effects of PM obtained from the rainy-warm and dry-cold seasons in five sites of Mexico City, and demonstrated that seasonal variability in PM composition strongly correlates with its potential to induce exposed cells to secrete TNFα and IL-6. Although other studies have identified PM related effects linked to season-related changes in composition (Bell et al. 2007; Cheung et al. 2011; Toscano et al. 2011; Traversi et al. 2010), our large number of samples allowed us to identify seasonally related changes in PM composition that explained the variation in the observed cellular responses.

Epidemiological studies have reported associations between seasonal increases in PM concentrations and adverse health outcomes, including mortality (Moolgavkar 2003; Smith et al. 2000). Increased PM levels are not the only determinant of toxic potential; undoubtedly,

chemical composition (Dergham et al. 2012; Totlandsdal et al. 2014) and seasonal changes in chemical composition play fundamental roles (Laden et al. 2000). Other epidemiological (Son et al. 2012) and experimental studies report evidence of seasonal biological effects in PM chemical composition (Becker et al. 2005; Camatini et al. 2012; Perrone et al. 2010). Specifically, a higher cell viability reduction was observed after exposure to PM collected in the summer vs. winter in two Italian cities; summer samples had a higher content of sulfates, Al, arsenic (As), Cr, Cu and Zn (Perrone et al. 2010). Similarly, another Italian study showed a greater cell toxicity and pro-inflammatory response after exposures to summer PM_{10} samples containing high levels of mineral dust elements, Fe and endotoxins vs. winter PM_{10} samples with lower content of the same constituents (Camatini et al. 2012).

We also observed seasonal differences in PM_{10} and $PM_{2.5}$ chemical composition. The anthropogenic related component (C_1: grouping V and PAHs) was higher in the PM samples from the winter (dry-cold season) that in the summer PM samples (wet-warm season) ($PM_{2.5}$>PM_{10}). A second identified component (C_2) grouped endotoxins and soil elements (e.g. Si, Al), showing opposite seasonal differences (higher in the summer than in the winter), mainly in PM_{10}.

Higher PM content of PAHs in the dry-cold season has been observed in previous studies performed in Mexico City (Mugica et al. 2010; Vega et al. 2011). However, no previous studies have described seasonal differences in PM-related PAHs content in Mexico City. Studies in other cities have documented higher PAHs levels during the coldest season (Perrone et al. 2010). Existing studies describing other seasonal-related changes in the chemical composition of PM from Mexico City are limited to the winter and early spring (Vega et al. 2011).

Seasonal differences in chemical composition are probably related to meteorological conditions (e.g. gas-partition ratios driven by temperature and humidity, windblown dust, thermal inversions, atmospheric mixing layer depth), and changes in human activities (e.g. traffic patterns) (Mugica et al. 2010; Pandolfi et al. 2014; Vega et al. 2011). Additionally, experimental evidence indicates that ozone co-existing in the atmosphere with wood smoke particles can decrease their PAHs content and bio-reactivity (Nordin et al. 2014). During the study period, we observed that the rainy-warm season had higher levels of ozone negatively correlating with PM-related PAHs content (data not shown). However, further research assessing the impact of atmospheric oxidative reactions on PM-related seasonal toxicity is required.

Pro-inflammatory potential of PM samples showed seasonal variation. Samples from the rainy-warm season were more potent than the samples from the dry-cold one and more potent for PM_{10} than $PM_{2.5}$. Pro-inflammatory cellular responses were positively correlated with the component in which soil constituents grouped (C_2). Other authors described a relation between pro-inflammatory PM potential and the PM content of endotoxins, Fe and Cu in samples collected in Europe (Guastadisegni et al. 2010) or endotoxins and carbon in samples from Netherlands (Steenhof et al. 2011). However, neither of those two studies analyzed seasonal variability.

The component (C_1) had a non-linear association with TNFα levels. TNFα responses showed an inverse U-shaped dose-response curve for C_1 scores. Samples with lower and higher C_1 content elicited a low TNFα secretion, whereas PM with intermediate C_1 content always elicited high TNFα secretion. Higher C_1 content mainly occurred in $PM_{2.5}$, specifically from the dry-cold season.

Seasonal patterns in cell responses related to PM PAHs content have been observed previously. Aung et al. (2011) described that PM rich in carbon, sulfates and Cu collected during the summer in California stimulated increased expression of genes related to inflammation, whereas PM collected in the winter showed a decreased inflammatory gene response but a higher PAHs content. Likewise, Camatini et al. (2012) reported that PM collected during the winter in Italy had ten times more PAHs and a decreased pro-inflammatory cell response than PM samples collected in the summer. However, they did not describe any effects related to interactions among PM components and PM fraction as we do in the present paper.

Our analyses indicated that PM-induced TNFα production decreases significantly as PAHs content in C_1 increases, after adjusting for PM-size and C_1+C_2 content. When the amount of PAHs present in C_1 reached ≥0.1 % of the C_1+C_2 content, PM-induced TNFα secretion was decreased. To our knowledge, this is the first report describing the relative participation of groups of PM constituents in PM-size and seasonally related cellular induction of TNFα secretion using real world particles. Larger content of soil components in the rainy-warm season PM_{10} related with a larger cytokine production, while larger PAHs content in $PM_{2.5}$ and PM_{10} dry-cold season samples was related with a lower TNFα production. The higher content of PAHs in $PM_{2.5}$ was linked with a pattern of lower cytokine induction when compared with PM_{10}.

Other reports support the idea that particle-mediated cell effects are related to particle constituents' interactions that alter physicochemical PM properties. Reduced bioavailability of PAHs when adsorbed to particles is one proposed mechanism (Goulaouic et al. 2008). Other researchers observed that *in vitro* PAHs-induced cell DNA damage signaling is more potent when PAHs are added in mixtures than when tested alone, and responses to mixtures containing

large PAHs were more persistent (Jarvis et al. 2013). Studies in zebrafish indicate that real-world PM rich in PAHs have increased embryotoxicity and dioxin-like activity than samples rich in mineral content (Mesquita et al. 2014). All these results are consistent with the evidence of PAHs-related and soil-related responses to PM from the present study.

Our data suggest that the mechanisms mediating TNFα and IL-6 production after PM stimulation are different. IL-6 production was low; more dispersed; had a weaker linear correlation with soil content and did not have a clear association pattern with PAHs content. These features suggest the existence of more complex interactions between PM components regulating the induction of IL-6 production, as well as modulatory interaction between TNFα and IL-6, as suggested by Raspe et al. (2013). They observed that exposure to various combinations of immunomodulatory amino acids altered TNFα or IL-6 expression after monocytes stimulation with LPS. Others describe differential interleukin response patterns when testing mixtures of PAHs adsorbed to particles on monocytic cells (Goulaouic et al. 2008).

Future studies are needed to fully understand how PM composition differentially affects signaling and cellular pathways, leading to different health outcomes related to seasonal PM composition. A more comprehensive evaluation of PM constituents and cellular responses would certainly increase our understanding of the mechanism involved (Dergham et al. 2012; Totlandsdal et al. 2014). This study centers on the complexity of PM composition. So far, we have explored two main groups/components of chemicals commonly identified in PM samples, without attempting to isolate specific constituents (e.g. Na, endotoxins) or constituents interactions within each component. We still lack knowledge on specific biological effects attributable to PM constituents. For example, on the simultaneous effects of PM-related PAHs'

and various cell outcomes like inflammation, biotransformation and DNA damage potential (Ovrevik et al. 2010; Teixeira et al. 2012). As previously postulated, PM-induced cell responses result from complex interactions among constituents of the PM mixture (Osornio-Vargas et al. 2011). This study provides a foundation for future mechanistic studies where larger sets of biological effects and PM constituents could be incorporated. Understanding cell-PM constituents' interactions is an important step bridging toxicological and epidemiological studies. Our results support the hypothesis that seasonal PM changes in composition impact the biological responses to PM. Our current epidemiological cohort study will further explore the role of seasonality of air pollution exposures during pregnancy and potential negative impacts on birth outcomes (O´Neill et al. 2013).

CONCLUSIONS

PM showed seasonal variations in composition and *in vitro* pro-inflammatory effects, strongly driven by constituents related to soil sources. However, soil-related elements in samples collected during the dry-cold season, which had higher PAHs content, did not trigger a pro-inflammatory response. For TNFα, secretion did not appear to be induced by exposure to PM when the PAHs content was ≥ 0.1%. However, IL-6 production did not fit this pattern, probably as a result of more complex interactions between PM components, PM-size and/or cytokine cross-talk. This should be considered in future toxicological and epidemiological studies linking PM composition with deleterious effects.

REFERENCES

Afifi A, Clark VA, May S. 2004. Computer-Aided Multivariate Analysis. 4th ed. U.S.A:Chapman and Hall/CRC.

Alfaro-Moreno E, Torres V, Miranda J, Martinez L, Garcia-Cuellar C, Nawrot TS, et al. 2009. Induction of IL-6 and inhibition of IL-8 secretion in the human airway cell line Calu-3 by urban particulate matter collected with a modified method of PM sampling. Environ Res 109:528-535.

Aung HH, Lame MW, Gohil K, He G, Denison MS, Rutledge JC, et al. 2011. Comparative gene responses to collected ambient particles in vitro: endothelial responses. Physiol Genomics 43:917-929.

Becker S, Dailey LA, Soukup JM, Grambow SC, Devlin RB, Huang Y-CT. 2005. Seasonal Variations in Air Pollution Particle-Induced Inflammatory Mediator Release and Oxidative Stress. Environ Health Perspect 113:1032-1038.

Bell ML, Dominici F, Ebisu K, Zeger SL, Samet JM. 2007. Spatial and Temporal Variation in PM2.5 Chemical Composition in the United States for Health Effects Studies. Environ Health Perspect 115:989-995.

Camatini M, Corvaja V, Pezzolato E, Mantecca P, Gualtieri M. 2012. PM10-biogenic fraction drives the seasonal variation of proinflammatory response in A549 cells. Environ Toxicol 27:63-73.

Cheung K, Daher N, Shafer MM, Ning Z, Schauer JJ, Sioutas C. 2011. Diurnal trends in coarse particulate matter composition in the Los Angeles Basin: J Environ Monit 13:3277-3287.

Dergham M, Lepers C, Verdin A, Billet S, Cazier F, Courcot D, et al. 2012. Prooxidant and proinflammatory potency o fair pollution particulate matter ($PM_{2.5-0.3}$) produced in rural, urban, or industrial surroundings in human bronchial epithelial cells (BEAS-2B). Chem Res Toxicol 25:904-919.

Diaz J, Tobias A, Linares C. 2012. Saharan dust and association between particulate matter and case-specific mortality: a case-crossover analysis in Madrid (Spain). Environmental Health: a global access science source 11:11. doi:10.1186/1476-069X-11-11.

Goulaouic S, Foucaud L, Bennasroune A, Laval-Gilly P, Falla J. 2008. Effect of polycyclic aromatic hydrocarbons and carbon black particles on pro-inflammatory cytokine secretion: impact of PAH coating onto particles. J Immunotoxicol 5:337-345.

Guastadisegni C, Kelly FJ, Cassee FR, Gerlofs-Nijland ME, Janssen NAH, Pozzi R, et al. 2010. Determinants of the Proinflammatory Action of Ambient Particulate Matter in Immortalized Murine Macrophages. Environ Health Perspect 118:1728-1734.

Hiraiwa K, van Eeden SF. 2013. Contribution of lung macrophages to the inflammatory responses induced by exposure to air pollutants. Mediators of inflammation 2013:619523.

INE-SEMARNAT (Instituto Nacional de Ecologia-Secretaría de Medio Ambiente y Recursos Naturales) 2011. Cuarto almanaque de datos y tendencias de la calidad del aire en 20 ciudades mexicanas (2000-2009). Mexico City, 95-113.

Jarvis IW, Bergvall C, Bottai M, Westerholm R, Stenius U, Dreij K. 2013. Persistent activation of DNA damage signaling in response to complex mixtures of PAHs in air particulate matter. Toxicol Appl Pharmacol 266:408-418.

Laden F, Neas LM, Dockery DW, Schwartz J. 2000. Association of fine particulate matter from different sources with daily mortality in six U.S. cities. Environ Health Perspect 108:941-947.

Legendre P, Legendre L. 2012. Numerical Ecology. 3rd English ed. Great Britain:Elsevier..

Manzano-Leon N, Quintana R, Sanchez B, Serrano J, Vega E, Vazquez-Lopez I, et al. 2013. Variation in the composition and in vitro proinflammatory effect of urban particulate matter from different sites. J Biochem Mol Toxicol 27:87-97.

Mesquita SR, van Drooge BL, Reche C, Guimaraes L, Grimalt JO, Barata C, et al. 2014. Toxic assessment of urban atmospheric particle-bound PAHs: relevance of composition and particle size in Barcelona (Spain). Environ Pollut 184:555-562.

Moolgavkar SH. 2003. Air pollution and daily mortality in two U.S. counties: season-specific analyses and exposure-response relationships. Inhal Toxicol 15:877-907.

Mugica V, Hernández S, Torres M, García R. 2010. Seasonal Variation of Polycyclic Aromatic Hydrocarbon Exposure Levels in Mexico City. J Air Waste Manag Assoc 60:548-555.

Nordin EZ, Uski O, Nyström R, Jalava P, Eriksson AC, Genberg J, et al. 2015. Influence of ozone initiated processing on the toxicity of aerosol particles from small scale wood combustion. Atmos Environ 102:282-289.

O'Neill MS, Osornio-Vargas A, Buxton MA, Sanchez BN, Rojas-Bracho L, Castillo-Castrejon M, et al. 2013. Air pollution, inflammation and preterm birth in Mexico City: study design and methods. Sci Total Environ 448:79-83.

Osornio-Vargas AR, Serrano J, Rojas-Bracho L, Miranda J, Garcia-Cuellar C, Reyna MA, et al. 2011. In vitro biological effects of airborne $PM_{2.5}$ and PM_{10} from a semi-desert city on the Mexico-US border. Chemosphere 83:618-626.

Ovrevik J, Arlt VM, Oya E, Nagy E, Mollerup S, Phillips DH, et al. 2010. Differential effects of nitro-PAHs and amino-PAHs on cytokine and chemokine responses in human bronchial epithelial beas-2b cells. Toxicol Appl Pharmacol 242:270-280.

Pandolfi M, Tobias A, Alastuey A, Sunyer J, Schwartz J, Lorente J, Pey J, Querol X. 2014. Effect of atmospheric mixing layer depth variations on urban air quality and daily mortality during Saharan dust outbreaks. Sci Total Environ 494-495:283-289.

Peng RD, Dominici F, Pastor-Barriuso R, Zeger SL, Samet JM. 2005. Seasonal analyses of air pollution and mortality in 100 US cities. Am J Epidemiol 161:585-594.

Perrone MG, Gualtieri M, Ferrero L, Porto CL, Udisti R, Bolzacchini E, et al. 2010. Seasonal variations in chemical composition and in vitro biological effects of fine PM from Milan. Chemosphere 78:1368-1377.

Pope CA, 3rd, Dockery DW. 2006. Health effects of fine particulate air pollution: lines that connect. J Air Waste Manag Assoc 56:709-742.

Raspe C, Czeslick E, Weimann A, Schinke C, Leimert A, Kellner P, et al. 2013. Glutamine and alanine-induced differential expression of intracellular IL-6, IL-8, and TNF-alpha in LPS-stimulated monocytes in human whole-blood. Cytokine 62:52-57.

SEDEMA, Secretaría del Medio Ambiente, Gobierno del Distrito Federal http://www.aire.df.gob.mx/default.php?opc=%27aKBhnmI=%27&opcion=Zw== [accessed 17 August 2015]

Shah PS, Balkhair T. 2011. Air pollution and birth outcomes: a systematic review. Environ Int 37:498-516.

Smith RL, Spitzner D, Kim Y, Fuentes M. 2000. Threshold Dependence of Mortality Effects for Fine and Coarse Particles in Phoenix, Arizona. J Air Waste Manag Assoc 50:1367-1379.

Son JY, Lee JT, Kim KH, Jung K, Bell ML. 2012. Characterization of fine particulate matter and associations between particulate chemical constituents and mortality in Seoul, Korea. Environ Health Perspect 120:872-878.

Steenhof M, Gosens I, Strak M, Godri KJ, Hoek G, Cassee FR, et al. 2011. In vitro toxicity of particulate matter (PM) collected at different sites in the Netherlands is associated with PM composition, size fraction and oxidative potential--the RAPTES project. Part Fibre Toxicol 8:26.

Teixeira EC, Pra D, Idalgo D, Henriques JA, Wiegand F. 2012. DNA-damage effect of polycyclic aromatic hydrocarbons from urban area, evaluated in lung fibroblast cultures. Environ Pollut 162:430-438.

Toscano G, Moret I, Gambaro A, Barbante C, Capodaglio G. 2011. Distribution and seasonal variability of trace elements in atmospheric particulate in the Venice Lagoon. Chemosphere 85:1518-1524.

Totlandsdal AI, Ovrevik J, Cochran RE, Herseth JI, Bolling AK, Lag A, et al. 2014. The occurrence of polycyclic aromatic hydrocarbons and their derivatives and the proinflammatory potential of fractionated extracts of diesel exhaust and wood smoke particles. J Environ Sci Health A Tox Hazard Subst Environ Eng 49:383-396.

Traversi D, Alessandria L, Schiliro T, Chiado Piat S, Gilli G. 2010. Meteo-climatic conditions influence the contribution of endotoxins to PM10 in an urban polluted environment. J Environ Monit 12:484-490.

Vega E, Ruiz H, Escalona S, Cervantes A, Lopez-Veneroni D, Gonzalez-Avalos E, et al. 2011. Chemical composition of fine particles in Mexico City during 2003-2004. Atmospheric Pollution Research, 2:477-483.doi:10.5094/APR.2011.054.

Zanobetti A, Austin E, Coull BA, Schwartz J, Koutrakis P. 2014. Health effects of multi-pollutant profiles. Environ Int 71:13-19.

Table 1. Summary statistics for PM-constituents according to type of constituent (endotoxin, PAHs, or elements), season, and PM-size.

	PM_{10}		$PM_{2.5}$	
Constituent[a]	**Median**	**Mean (95% CI)**	**Median**	**Mean (95% CI)**
Endotoxin				
Dry-cold	4.6	5.2 (4.5, 5.8)	2.1*	2.3 (1.6, 3.0)
Rainy-warm	4.1	6.6 (4.1, 9.2)	0.5*	1.0 (0.4, 1.5)
PAHs				
Dry-cold	47.9	53.4 (42.2, 64.7)	56.7*	71.1 (57.3, 84.8)
Rainy-warm	41.7	37.1 (31.4 , 42.8)	24.2*	30.7 (23.0, 38.5)
Elements				
Dry-cold	71,183.4*	68,391.6 (64978, 71805)	45,683.7*	46,529.1 (39870, 53188)
Rainy-warm	119,909.3*	126,206.4 (105260, 147153)	20,376.5*	27,657.9 (17820, 37496)

[a] ng/mg p

* indicates significant differences between seasons by comparing same PM-size constituents ($p < 0.05$; Mann-Whitney test)

CI=Confidence Interval

Table 2. Summary statistics for cytokine production (pg/mL) in response to PM exposures according to PM size and season.

	PM_{10}			$PM_{2.5}$		
Cytokine[a]	**Median**	**Mean (95% CI)**	**CV(%)**	**Median**	**Mean (95% CI)**	**CV(%)**
TNFα						
Dry-cold	60.52*	65.08 (59.03, 71.12)	22.50	5.22*	8.42 (5.23, 11.62)	91.80
Rainy-warm	98.60*	105.63 (79.90, 131.35)	52.02	14.01*	32.24 (13.31, 51.17)	125.40
IL-6						
Dry-cold	0.51*	0.62 (0.37, 0.87)	96.93	0.01*	0.22(-0.001, 0.44)	243.80
Rainy-warm	3.67*	3.89 (2.81, 4.97)	59.41	0.31*	1.01 (0.29, 1.72)	151.20

[a] pg/mL, after subtracting cytokine levels produced by non-exposed control cells.

* indicates significant differences between seasons by comparing same cytokine per PM-size ($p < 0.05$; Mann-Whitney test)

CV=Coefficient of Variation

CI=Confidence Interval

Figure 1. C_1, C_2 component scores plot according to PM-size and season. Samples (n=90) according to season and PM-size (groups) are well differentiated in the content of C_1 and C_2. Rainy-warm PM_{10} had high values of C_2 and average values of C_1; Dry-cold PM_{10} presented average C_1 values and moderate high values of C_2; Rainy-warm $PM_{2.5}$ generally had low values of C_1 and C_2; Dry-cold $PM_{2.5}$ showed high values of C_1 and low values of C_2. The zero in both axes corresponds to the mean of all samples, while integer values are based on the observation´s component loading and the standardized value of the original variable, summed over all variables.

Figure 2. Scatter plots presenting cytokine responses (ln) and PM C_1 and C_2 component scores. Patterns of association between TNFα and C_1 and C_2 were seen. **2A**) Low levels of PM-induced TNFα occurred with very low and very high values of C_1, corresponding to $PM_{2.5}$ samples from rainy-warm and dry-cold seasons, respectively. The overall correlation was negative (r=-0.32; p=0.002). **2B)** C_2 and TNFα values show a significant positive correlation (r=0.65; p<0.001). **2C and 2D**) shows highly dispersed low correlation patterns of IL-6 with C_1 (r=-0.36; p=0.000) and C_2 (r=0.50; p=0.000). Cytokine values after subtracting cytokine levels produced by non-exposed control cells. The zero in the X-axis correspond to the mean of all samples, while integer values are based on the observation´s component loading and the standardized value of the original variable, summed over all variables.

Figure 3. Scatter plot of PM-induced TNFα vs. the percentage of PM C_1-related PAHs (A). The relationship between TNFα (ln) and PM C_1-related PAHs was negative (adjusted R-squared = 0.75; Prob > F = 0.000). (B). Untransformed data shows, that TNFα production is

markedly reduced when C_1-related PAHs is greater than ~0.1%, or C_1+C_2 content is low. Data points are marked by the category of PM C_1+C_2 content in quartiles.

Figure 1.

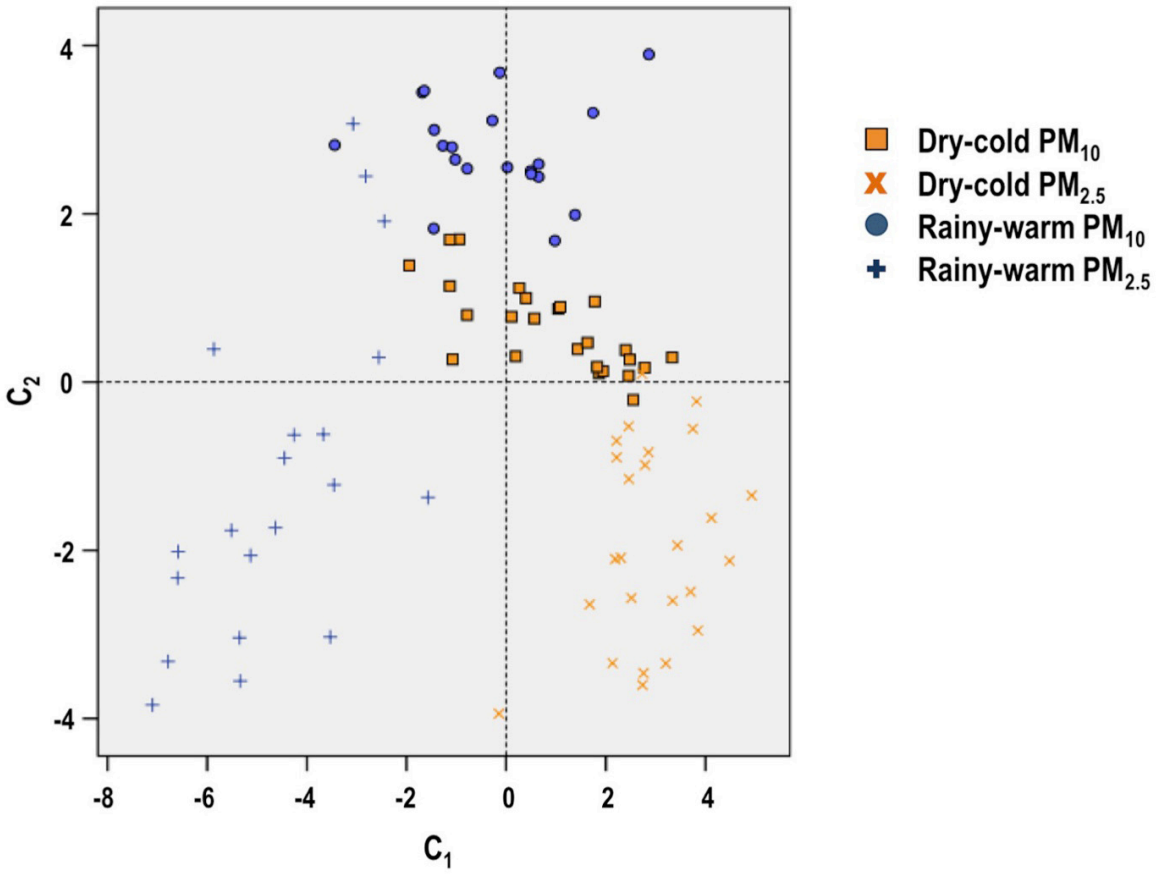

4
2
0
-2
-4
-8
-6
-4
-2
0
2
4
C2
C1
Dry-cold PM10
Dry-cold PM2.5
Rainy-warm PM10
Rainy-warm PM2.5

Figure 2.

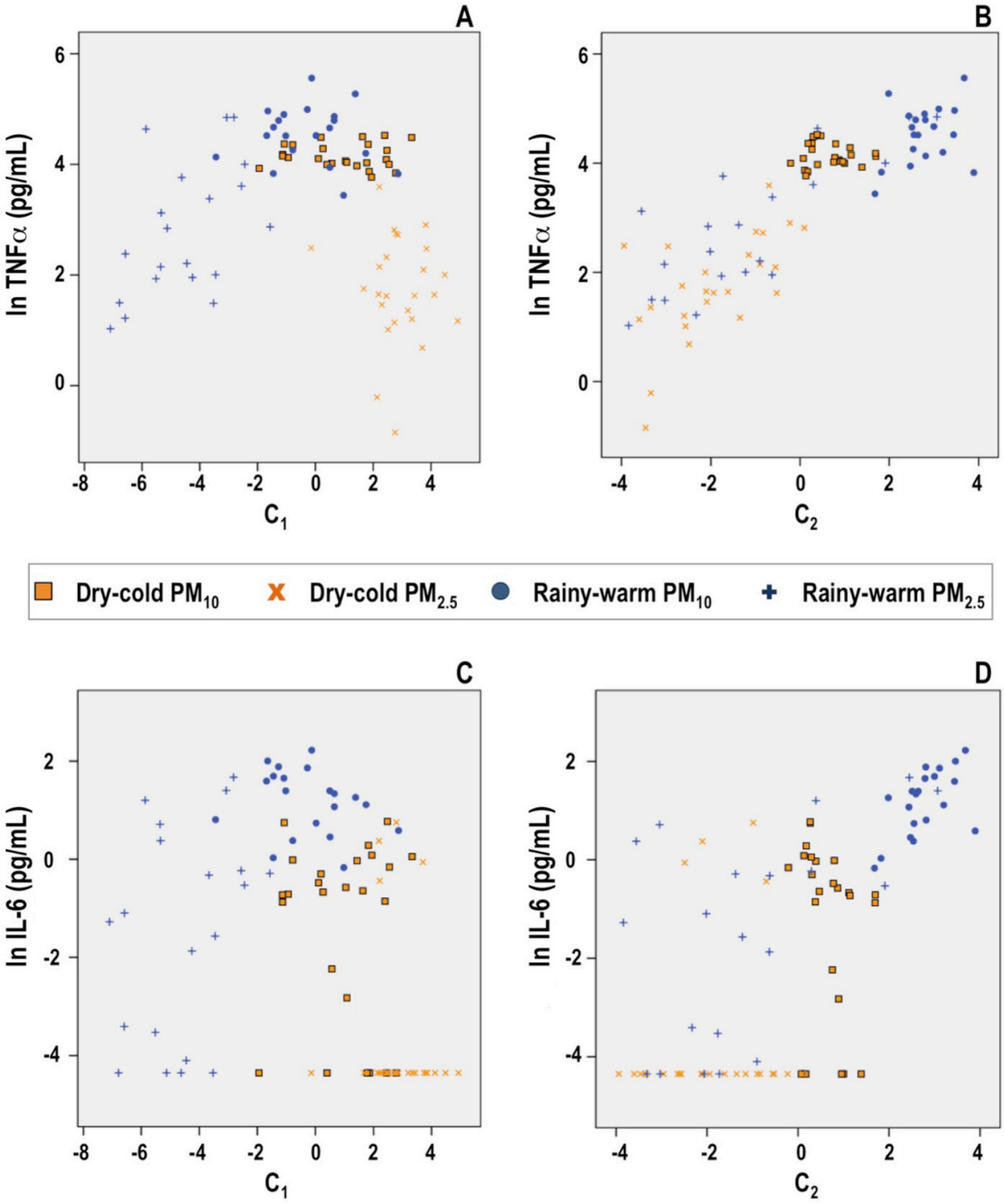
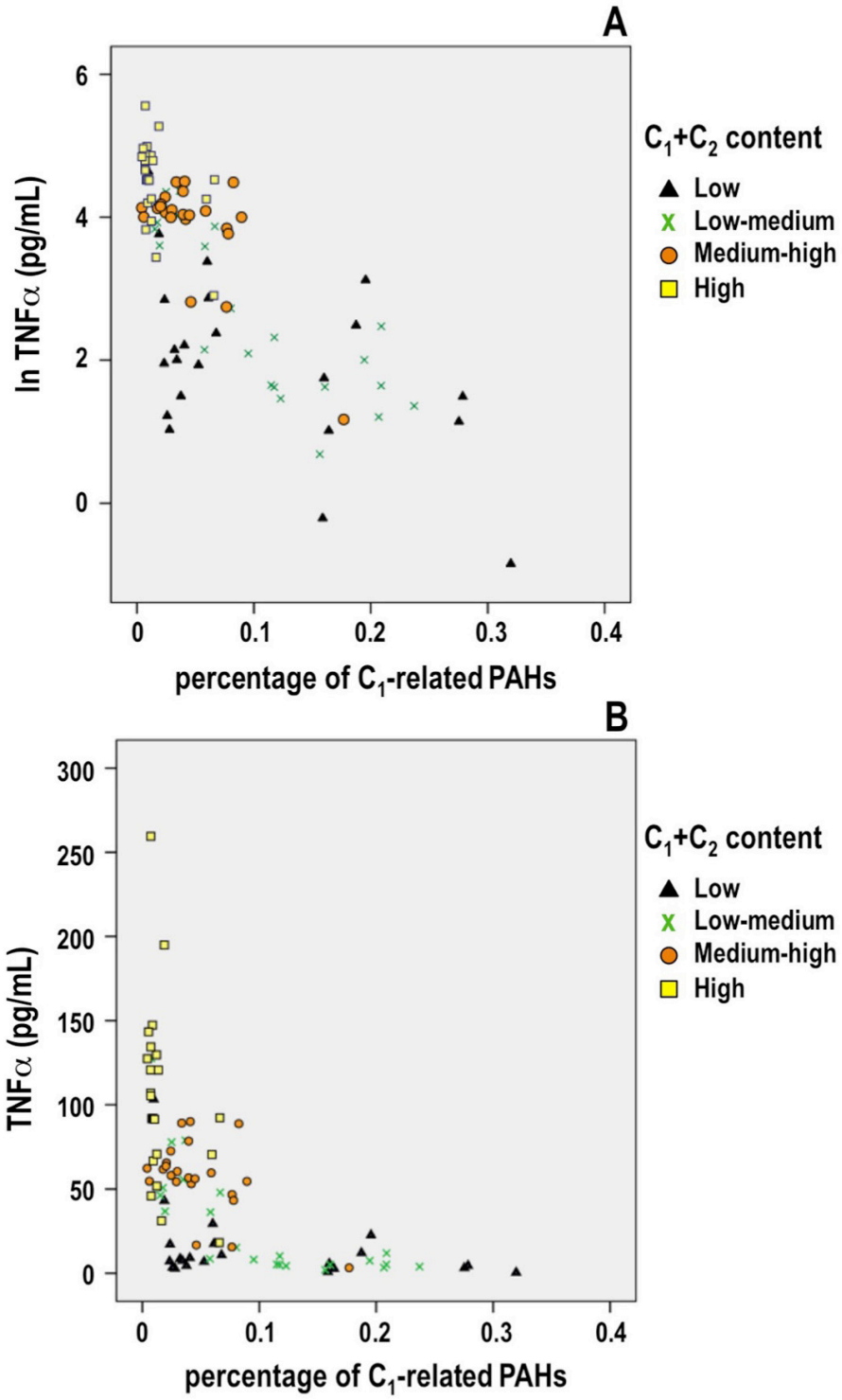

A
B
C
D
ln TNFα (pg/mL)
ln IL-6 (pg/mL)
C1
C2
Dry-cold PM10
Dry-cold PM2.5
Rainy-warm PM10
Rainy-warm PM2.5

Figure 3.


A
ln TNFα (pg/mL)
6
4
2
0
0
0.1
0.2
0.3
0.4
percentage of C1-related PAHs
C1+C2 content
Low
Low-medium
Medium-high
High
B
TNFα (pg/mL)
300
250
200
150
100
50
0
0
0.1
0.2
0.3
0.4
percentage of C1-related PAHs
C1+C2 content
Low
Low-medium
Medium-high
High